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SMAD1 promoter hypermethylation and lack of SMAD1 expression in Hodgkin lymphoma: a potential target for hypomethylating drug therapy

Running head: SMAD1 hypermethylation in Hodgkin Lymphoma

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Hodgkin lymphoma (HL) is an immunologically active lymphoid neoplasm composed of a few (usually 1-10%) neoplastic Hodgkin and Reed-Sternberg (HRS) cells or lymphocyte predominant (LP) cells and >90% non-neoplastic cells, mainly T- and B-lymphocytes, plasma cells, macrophages, eosinophils and fibroblasts. The substantial amount of reactive cells in HL is supposed to be the net effect of a complex network of cytokines and chemokines secreted by either the HRS cells or non-neoplastic cells (1). One component of this network is transforming growth factor beta (TGF- β), which is produced by HRS cells and cancer-associated fibroblasts. TGF- β unfolds its immunosuppressive impact by stimulating tumor-infiltrating T-lymphocytes (TIL) to differentiate into anergic, tumor-promoting, regulatory T-cells (Tregs) (2). Additionally, TGF- β inhibits natural killer (NK) cells - one of the key components of innate anticancer immunity (3). Interestingly and still poorly understood, the HRS cells themselves seem to remain unaffected by the tumor-suppressive properties of TGF- β (4).

Recent studies on diffuse large B-cell lymphoma (DLBCL) revealed a previously unknown tumor-suppressive signaling axis involving SMAD1 as a downstream messenger of TGF- β (5). SMAD1 functions as an intracellular signal transducer between extracellular TGF- β and the nucleus, where it modulates the transcription of target genes. This signaling cascade was shown to be recurrently inactivated in DLBCL, mainly by hypermethylation of five promoter regions surrounding the *SMAD1* transcription start site, which finally generates a significant growth advantage for lymphoma cells (5). In the course of these investigations, we noted that SMAD1 was not expressed in HRS cells of screened HL cases. This led us to hypothesize that the absence of SMAD1 expression in HRS cells may mechanistically be linked to their resistance to the tumor-suppressive effects of TGF- β (4).

In order to further elucidate this finding, we analyzed 132 well-characterized archival tissue-microarrayed cases (6), eleven conventional routine lymphadenectomy specimens of patients suffering from all classic HL subtypes [77 nodular scleroses (NS); 48 mixed-cellularity (MC); 7 lymphocyte-rich (LR); 5 lymphocyte depleted; and 6 unclassifiable classic HL] and fourteen routine samples of patients suffering from nodular lymphocyte predominant HL (NLPHL) regarding the immunohistochemical expression of SMAD1. Importantly, to guarantee retained antigenicity, only

cases containing (physiologically) SMAD1 positive endothelia were considered. We found that the all NLPHL (14/14 cases; 100%) and the great majority of classic HL (138/143 cases; 97%; Figure 1 A-B) displayed SMAD1 negative LP and HRS cells, respectively. Only in five cases, single HRS cells faintly stained for SMAD1 (2 NS; 2 MC; and 1 LR classic HL). With respect to non-neoplastic cells, 65/143 classic HL (45%) showed moderate (15-49% of TIL) up to abundant ($\geq 50\%$ of TIL) amounts of SMAD1 positive surrounding TIL, thus being potentially susceptible to the suppressive influence of TGF- β (Figure 1 A-B); in NLPHL, 11/14 (79%) instances displayed abundant SMAD1 expressing TIL, including TIL involved in resetting around LP cells (Suppl. Figure 1). The presence of abundant SMAD1 expressing TIL did not correlate with disease stage, patients' age, gender, presence of B-symptoms, EBV-association or outcome, while showing significant correlation with the NS subtype (45/77 NS cases, i.e. 58%, compared to 20/66 non-NS cases, i.e. 30%, $p^{\text{Chi-square}}=0.025$) and with the amount of FOXP3-positive Tregs (Rho=0.351, $p^{\text{Spearman}}=0.000053$), which both, in turn, may directly be linked to the effects of TGF- β , promoting sclerosis and a shift towards Treg differentiation (2). In contrast, surrounding plasma cells seemed to lack SMAD1 expression, potentially rendering them insensitive for the pro-apoptotic and anti-proliferative signals of TGF- β (7). With regard to plasma cells, this largely fits to the newly described negative prognostic impact of their increased numbers in classic HL (8).

To further strengthen our hypothesis, the promoter methylation status of the *SMAD1* gene was investigated in six different HL cell lines, including one NLPHL cell line (DEV) exactly as described (5). Methylation analysis by bisulfite sequencing was successful for three regions of the *SMAD1* promoter and yielded four hypermethylated cell lines (L428, KMH-2, DEV, HDLM-2), which substantially differed particularly regarding their methylation of region A4(3) from cell lines showing no evidence for promoter hypermethylation (L1236, L540; Figure 2A). Importantly, KMH-2 is known to be *SMAD1* p.ADTP220fs mutant https://portals.broadinstitute.org/ccle/page?cell_line=KMH2_haematopoietic_and_lymphoid_tissue, which additionally points towards a potential role of this gene silencing in lymphomagenesis.

The impact of the promoter methylation status of *SMAD1* on protein expression was further addressed by Western blot analysis, comparing one hypermethylated cell line (DEV) with a cell line without hypermethylation (L1236). Concordantly, the expression of SMAD1 differed clearly without detectable protein in the DEV cell line (Figure 2B). When treated with the DNA methyltransferase (DNMT) inhibitor decitabine (DAC), which reverses, among others, the hypermethylation of the *SMAD1* promoters, the SMAD1-negative cell line DEV immediately died after exposure. As expected, the expression of SMAD1 was not affected by treatment in the L1236 cell line that is not hypermethylated.

To obtain further clinical evidence, the promoter methylation status of *SMAD1* was assessed on three classic HL patient samples. For this purpose, we used our newly developed, flow sorting–assisted technique for HRS cell enrichment from formalin-fixed and paraffin-embedded tissues allowing for targeted genetic analysis of DNA isolated from classic HL tumor cells (9). In this collective, not only the A4(3) promoter region of *SMAD1*, but also the A1(1) region was substantially hypermethylated (Figure 2C). Furthermore, *SMAD1* promoter hypermethylation was identified in sorted tumor-infiltrating plasma cells, fitting to the immunohistochemically noticed lack of SMAD1 in plasma cells.

Although risk-adjusted standard treatment of HL is successful in over 90% of patients, relapses after salvage therapy and refractory cases represent oncological challenges and run an unfavorable clinical course with limited therapeutic options. In this context, demethylating agents as DAC and azacytidine may be of potential therapeutic interest and have already shown promising effects. At clinically relevant concentrations, DAC has been documented to inhibit the growth of classic HL cell lines *in vitro* and a single case observation of regressing relapsed classic HL as an unexpected “side effect” of azacytidine has been reported in a patient suffering from concomitant myelodysplastic syndrome (10, 11). DAC and azacytidine inhibit DNMTs, and therewith reverse promoter hypermethylation of *SMAD1* (12). Importantly, promising results with DAC were obtained in DLBCL cell lines lacking SMAD1 expression due to promoter hypermethylation too (5). Only four days of treatment were

sufficient to restore *SMAD1* transcription and protein expression in a subset of initially *SMAD1* negative DLBCL cell lines, which was corroborated by observations in a patient-derived xenograft DLBCL mouse model with proven *SMAD1* promoter hypermethylation (5). Analogous to this, we treated the *SMAD1*-negative HL cell line DEV with DAC. Indeed, immediately after exposure, the cells died, possibly due to marked responsiveness towards DAC.

Since therapeutic reversion of *SMAD1* promoter hypermethylation would be of potential relevance only in the presence of TGF- β receptors (TGFB β), we analyzed publically available [Gene Expression Omnibus (GEO) accession no. GSE12453] and own (GEO accession no. GSE147387) gene expression data of primary HRS and LP cells and cell lines to estimate whether HRS and LP cells express TGFB β and associated proteins (ACVR1, ACVR1B, ACVR1C, ACVR2A, ACVR2B, ACVRL1, AMHR2, BMPR2, TGFB β 1, TGFB β 2, TGFB β 3, TGFB β AP1). The classic HL cell line KMH-2 contained relevant transcript levels of all TGFB β types, HDLM-2 expressed TGFB β 1 and TGFB β 3, and the NLPHL cell line DEV – TGFB β 1; the classic HL cell lines L1236 and L428 exhibited TGFB β AP1 transcripts.

In summary, our data suggest a likely important, not yet described role of *SMAD1* hypermethylation in HL potentially causing an imbalance of the TGF- β signaling axis responses in involved tissues. *SMAD1* has been demonstrated to be part of the TGF- β -mediated anti-proliferative pathway in different B-cell lymphomas. Intriguingly, lymphomas with mutated or knocked-out *SMAD1* were protected from the tumor-suppressive effects of TGF- β (13).

Lack of *SMAD1* expression in HRS and LP cells due to promoter hypermethylation or gene mutation may analogously contribute to their resistance towards the pro-apoptotic and anti-proliferative effects of TGF- β , despite the presence of TGFB β transcripts. This hypothesis is further supported by observations in EBV-positive classic HL, in which decreased *SMAD2* levels due to EBNA1-mediated increased protein turnover (14) disable TGF- β signaling, being congruent to our data regarding *SMAD1* downregulation in HRS cells.

In contrast, retained SMAD1 expression in surrounding TIL may be contributive to immune escape, as intact TGF- β signaling promotes T-cell differentiation into tumor-supporting Tregs (2), which is reflected by the observed correlation between higher amounts of FOXP3 positive Tregs and expression of SMAD1 in TIL. The connection between the tumor-suppressive effects of TGF- β on TIL has recently been a subject of a promising clinical study in which the infusion of TGF- β insensitive T-cells was successfully used in patients with EBV-positive relapsed classic HL (15).

Our data suggest a possible rationale for the application of a more tailored treatment with hypomethylating agents in HL that may be worth of prospective investigations, as respective agents such as DAC already showed promising results in *SMAD1* hypermethylated DLBCL (5) and in classic HL cell lines (10).

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Contribution of each author:

Magdalena M. Gerlach wrote the manuscript and evaluated the histology and immunohistochemical stains

Anna Stelling-Germani supervised *SMAD1* promoter methylation assessment, performed cell line viability experiments, corrected the manuscript and wrote the legend to Figure 2

Cheuk Ting Wu assessed *SMAD1* promoter methylation

Sebastian Newrzela provided gene expression data of Hodgkin lymphoma cell lines

Claudia Döring provided and analyzed gene expression data of Hodgkin lymphoma cell lines

Visar Vela enriched Hodgkin and Reed-Sternberg cells from archival clinical samples and isolated DNA from them

Anne Müller supervised *SMAD1* promoter methylation assessment

Sylvia Hartmann provided cell lines

Alexandar Tzankov designed the study, supervised histopathologic assessment, performed statistics, analyzed gene expression data of Hodgkin lymphoma cell lines, partially wrote and completely edited the manuscript

References

1. Poppema S. Immunobiology and pathophysiology of Hodgkin lymphomas. *Hematology Am Soc Hematol Educ Program*. 2005;231-238.
2. Liu Y, Zhang P, Li J, Kulkarni AB, Perruche S, Chen W. A critical function for TGF-beta signaling in the development of natural CD4+CD25+Foxp3+ regulatory T cells. *Nat Immunol*. 2008;9(6):632-640.
3. Chiu J, Ernst DM, Keating A. Acquired Natural Killer Cell Dysfunction in the Tumor Microenvironment of Classic Hodgkin Lymphoma. *Front Immunol*. 2018;9:267.
4. Menter T, Tzankov A. Lymphomas and Their Microenvironment: A Multifaceted Relationship. *Pathobiology*. 2019;86(5-6):225-236.
5. Stelling A, Wu CT, Bertram K, et al. Pharmacological DNA demethylation restores SMAD1 expression and tumor suppressive signaling in diffuse large B-cell lymphoma. *Blood Adv*. 2019;3(20):3020-3032.
6. Tzankov A, Matter MS, Dirnhofer S. Refined prognostic role of CD68-positive tumor macrophages in the context of the cellular microenvironment of classical Hodgkin lymphoma. *Pathobiology*. 2010;77(6):301-308.
7. Lebman DA, Edmiston JS. The role of TGF-beta in growth, differentiation, and maturation of B lymphocytes. *Microbes Infect*. 1999;1(15):1297-1304.
8. Gholiha AR, Hollander P, Hedstrom G, et al. High tumour plasma cell infiltration reflects an important microenvironmental component in classic Hodgkin lymphoma linked to presence of B-symptoms. *Br J Haematol*. 2019;184(2):192-201.
9. Juskevicius D, Jucker D, Dietsche T, et al. Novel cell enrichment technique for robust genetic analysis of archival classical Hodgkin lymphoma tissues. *Lab Invest*. 2018;98(11):1487-1499.
10. Swerev TM, Wirth T, Ushmorov A. Activation of oncogenic pathways in classical Hodgkin lymphoma by decitabine: A rationale for combination with small molecular weight inhibitors. *Int J Oncol*. 2017;50(2):555-566.
11. D'Alò F, Leone G, Hohaus S, et al. Response to 5-azacytidine in a patient with relapsed Hodgkin Lymphoma and a therapy-related myelodysplastic syndrome. *Br J Haematol*. 2011;154(1):141-143.
12. Foulks JM, Parnell KM, Nix RN, et al. Epigenetic drug discovery: targeting DNA methyltransferases. *J Biomol Screen*. 2012;17(1):2-17.
13. Munoz O, Fend F, de Beaumont R, Husson H, Astier A, Freedman AS. TGFbeta-mediated activation of Smad1 in B-cell non-Hodgkin's lymphoma and effect on cell proliferation. *Leukemia*. 2004;18(12):2015-2025.
14. Wood VH, O'Neil JD, Wei W, Stewart SE, Dawson CW, Young LS. Epstein-Barr virus-encoded EBNA1 regulates cellular gene transcription and modulates the STAT1 and TGFbeta signaling pathways. *Oncogene*. 2007;26(28):4135-4147.
15. Bollard CM, Tripic T, Cruz CR, Dotti G, Gottschalk S, Torrano V. Tumor-specific T cells engineered to overcome tumor immune evasion induce clinical responses in patients with relapsed Hodgkin lymphoma. *J Clin Oncol*. 2018;36(11):1128-1139.

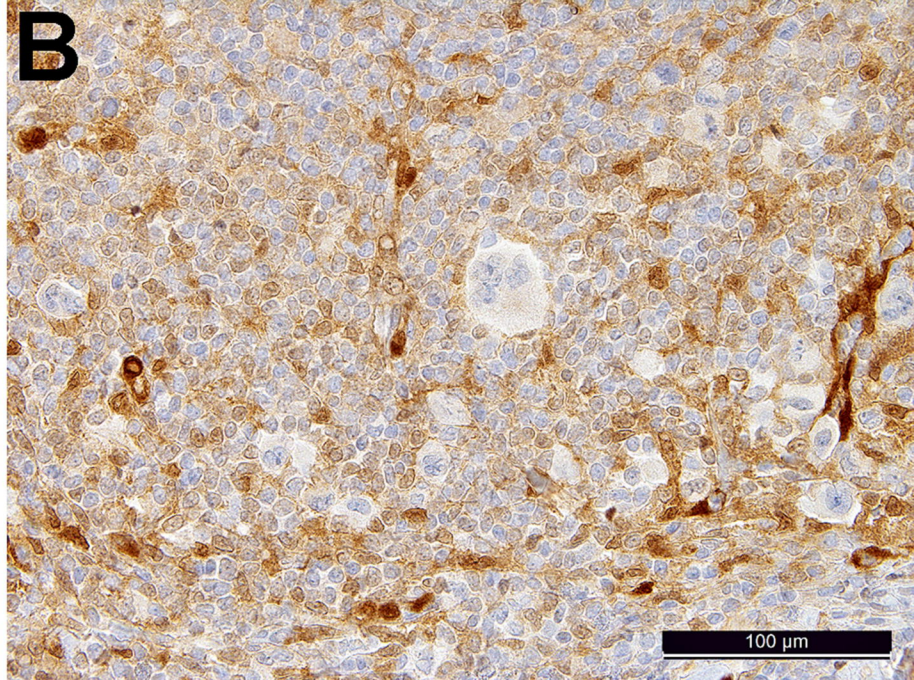
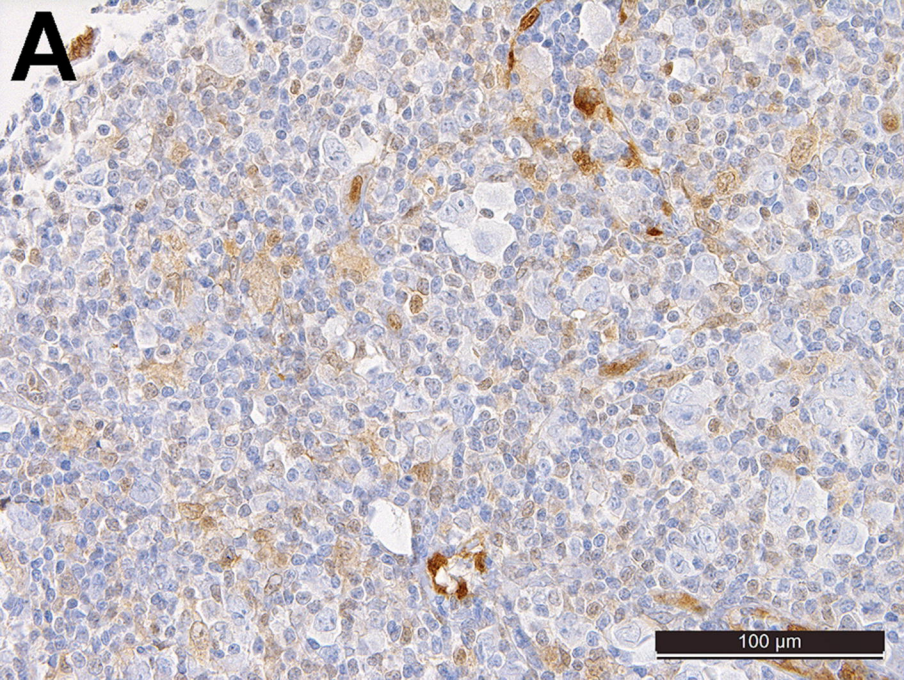
Figure legends

Figure 1: Expression of SMAD1 in classic Hodgkin lymphoma (cHL)

A: Tissue microarrayed archival mixed cellularity cHL with moderate amounts of SMAD1 positive tumor-infiltrating lymphocytes (TIL) and a few strongly staining endothelia. Note that all Hodgkin and Reed-Sternberg (HRS) cells are negative. **B:** Diagnostic lymphadenectomy of a nodular sclerosis cHL with abundant SMAD1 positive TIL and a few strongly staining endothelia. Note that all HRS cells are negative. Immunoperoxidase staining, magnification 400x.

Figure 2: The *SMAD1* promoter region is hypermethylated in Hodgkin lymphoma (HL) cell lines and patient samples.

A: Methylation analysis by bisulfite sequencing of region A1(1), A4(3) and B2(5) within the *SMAD1* promoter in a panel of HL cell lines. Each circle represents one CG dinucleotide; black circles indicate methylated, white circles indicate unmethylated cytosines. Each line represents one clone, 2-3 clones were sequenced per sample. X indicates aligned mismatches between genomic and bisulfite sequences. The cell line KMH-2 has a known frame-shift mutation at the *SMAD1* locus **B:** SMAD1 expression at the protein level after 0 (-) or 1 (+) μ M decitabine (DAC) treatment for 96 hours of two HL cell lines as determined by Western blotting. Results from two independent experiments are exemplified. DAC-treated samples of the DEV cell line are not shown due to lack of protein after demethylating treatment. **C:** Methylation analysis by bisulfite sequencing of region A1(1), A4(3) and B2(5) within the *SMAD1* promoter in three classic HL patient samples. Each circle represents one CG dinucleotide; black circles indicate methylated, white circles indicate unmethylated cytosines. Each line represents one clone, 2-3 clones were sequenced per sample. X indicates aligned mismatches between genomic and bisulfite sequences.



A

A
L1236

A1 (1)

A4 (3)

B2(5)

L428

KMH-2

DEV

HDLM-2

L540

C

Pat1

Pat2

Pat3

B

DEV

L1236

DAC

SMAD 1

Tubulin

—

—

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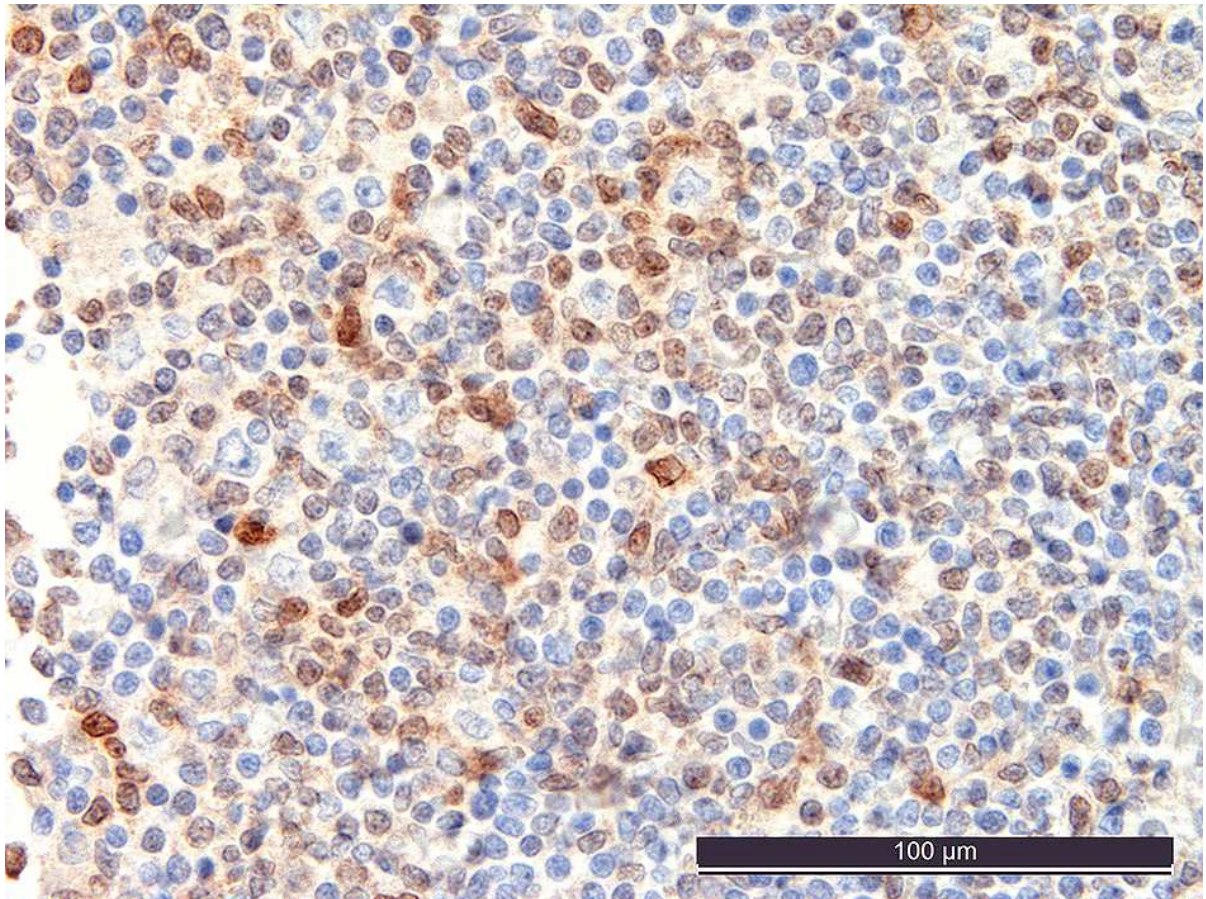
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Western blot analysis showing p38 phosphorylation in H1299 cells. The blot displays six lanes corresponding to the treatments: control, IL-1, IL-1 + SB, IL-1 + SB + J, IL-1 + J, and IL-1 + J + SB. The bands represent p38 phosphorylation, with the most intense band observed in the IL-1 + J lane, indicating maximal phosphorylation.



Suppl. Figure 1: Expression of SMAD1 in nodular lymphocyte predominant (LP) Hodgkin lymphoma

Substantial amounts of SMAD1 positive tumor-infiltrating lymphocytes including such that rim the neoplastic LP cell. Note that the large LP cells, including the rimmed one, are negative.

Immunoperoxidase staining, magnification 400x.